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(54) Title: FACTOR VIII PURIFICATION PROCESS**(57) Abstract**

There is provided in accordance with the practice of this invention a process for separating Factor VIII complex from an impure protein fraction containing Factor VIII complex. An aqueous solution of the impure protein fraction containing Factor VIII complex is applied to a heparin-coupled chromatographic medium, to bind the Factor VIII complex to the medium. The factor VIII is then recovered from the heparin-coupled chromatographic medium by elution with an aqueous solution comprising CaCl₂ and histidine. The recovered Factor VIII is further purified by precipitation with a solution comprising glycine and NaCl, and washing the resultant precipitate with a solution comprising histidine, glycine, and NaCl to provide a Factor VIII complex solution with a specific activity of about 70 to about 150 units/mg.

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FACTOR VIII PURIFICATION PROCESS

Field of the Invention

This invention relates to an improved process for preparing Factor VIII concentrates.

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Background of the Invention

Coagulation of blood is a complex process requiring the sequential interaction of a large number of components, nearly all of which are proteins. These 20 components include fibrinogen and Factors II, V, VII, VIII, IX, X, XI, and XII. A lack of any of these components, or a nonfunctional component, can lead to an inability of the blood to clot when required, with resultant excessive and life-threatening blood loss to 25 the patient.

Factor VIII is absent or is present at deficient levels in certain individuals. For example, persons who have a deficiency (or absence) of Factor VIII, i.e., persons suffering from hemophilia A, have blood 30 which either fails to clot or clots only after longer periods of time than the time required for clotting in a person who has a normal level of Factor VIII.

Factor VIII is present in plasma as a high-molecular-weight complex (Factor VIII complex), which 35 includes Factor VIII:C and von Willebrand factor (Factor VIII:RAG or vWF). Factor VIII:C promotes blood coagulation. Factor VIII:RAG interacts with platelets

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1 t promote aggregation of the platelets and, when
incorporated in the Factor VIII complex, acts as a
stabilizer for Factor VIII:C.

5 The primary therapeutic use of Factor VIII has
been its intravenous administration to hemophilia A
patients. In severe cases, relatively high concen-
trations of Factor VIII are required. These high
concentrations are obtained by purification and
concentration of Factor VIII. However, purification
10 often leads to instability and loss of Factor VIII:C
activity because of the removal of Factor VIII:RAG from
the Factor VIII complex during purification. Thus, the
resultant purified product is a mixture of both stable
Factor VIII complex and unstable Factor VIII:C, along
15 with contaminating proteins that have not been removed.
Since these solutions contain an undesirably large
portion of contaminating and inactive proteins, and
since only Factor VIII:C is effective in treating
20 hemophilia A patients, larger amounts of proteins have
to be infused into patients than would be required if
all the purified protein were active Factor VIII:C.

25 Some processes for producing Factor VIII
concentrate have been based on a discovery by Poole et
al. (Nature, 203, p. 312 (1964)) that the precipitate
remaining after plasma is frozen and then thawed, i.e.,
the cryoprecipitate, contains Factor VIII in a
concentrated form and excludes various other protein
fractions. It was discovered that, in addition to
Factor VIII, the cryoprecipitate also includes the
30 major portion of the fibronectin component of plasma.

35 Work progressed over the years to perfect the
separation of Factor VIII from other proteins in the
cryoprecipitate, including the fibronectin component,
so that the resulting products would incorporate
increased concentrations of Factor VIII relative to the
other proteins present in the plasma.

One area in which a substantial amount of work has

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1 been done in the production of Factor VIII is based on
the use of polyethylene glycol (PEG) to precipitate
Factor VIII from other proteins in an aqueous
cryoprecipitate solution. For example, U.S. Patent No.
5 3,652,530, which issued on March 28, 1972 to A. J.
Johnson, discloses a process for preparing a Factor
VIII concentrate by fractionating cryoprecipitate with
PEG at a relatively lower concentration to precipitate
fibrinogen and other proteins, and then increasing the
10 PEG concentration to precipitate Factor VIII.

U.S. Patent No. Re. 29,698 to Fekete et al.
discloses a process for production of Factor VIII by
which heparin is added to a cryoprecipitate solution,
along with PEG, to provide increased yields of
15 precipitated Factor VIII. It is disclosed that the
amount of heparin employed during the fractionation
step can vary, with the optimum concentration being one
unit of heparin per ml of the plasma solution, whereas
concentrations of heparin greater than about 10 units
20 per ml are to be avoided as dangerous.

Purification of Factor VIII by chromatography on
heparin (European Patent Application. No. 90308104.0 to
Battacharya et al. and Madras et al., Haemostasis, 7,
321-331 (1978)) has also been used. The Madras
25 process, while producing Factor VIII complex, results
in a Factor VIII complex with little or no activity.
The Battacharya process results in an active Factor
VIII; however, the specific activity of the protein
purified was at best 66 units/mg. (The phrase
30 "specific activity" as used herein means units of
Factor VIII:C clotting activity per milligram of
protein. A "unit" is defined as the amount of Factor
VIII:C in one ml of normal plasma.)

Purification of Factor VIII has also been achieved
35 by chromatography on monoclonal antibody-containing
chromatography media (U.S. Patents Nos. 4,361,509 and
Re. 32,001 to Zimmerman et al.). Such procedures

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1 result in Factor VIII:C of very high specific activities, approximately 1500 to 2500 units/mg of total protein, and, therefore, high purity. However,
5 in the Zimmerman process, the Factor VIII:R_{Ag} is dissociated from Factor VIII:C, which results in the Factor VIII:C being unstable.

Currently, the methods used to purify Factor VIII result in protein preparations, i.e., Factor VIII concentrates, which have a relatively low Factor VIII:C specific activity. It is desirable that there be provided an improved process for the separation of Factor VIII complex, i.e., the intact Factor VIII:C/Factor VIII:R_{Ag} complex, from contaminating proteins for producing Factor VIII which results in increased purity, higher concentration, and enhanced yields and stability of the protein.

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1 Summary of the Invention

The present invention is directed to a process for separating Factor VIII complex from an impure protein fraction containing Factor VIII complex, for example, from a plasma fraction or from any recombinant-DNA or transgenic-derived materials containing Factor VIII complex. The Factor VIII complex is separated by applying the impure protein fraction to a heparin-coupled chromatographic medium, binding Factor VIII complex to the heparin, and then eluting the Factor VIII complex from the chromatographic medium using an aqueous solution comprising CaCl₂ and histidine. The Factor VIII complex is further purified by precipitating the Factor VIII complex from the eluate with glycine and sodium chloride and washing the precipitate with a wash solution. The Factor VIII complex precipitate is then dissolved in an aqueous solution to provide a Factor VIII complex solution with a specific activity of about 100 to about 150 units/mg.

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1 Brief Description of the Drawing

These and other features and advantages of the present invention will become better understood with reference to the following description, appended 5 claims, and accompanying drawings, wherein:

FIG. 1 is a flow chart illustrating an exemplary embodiment of a process provided in accordance with practice of the present invention for preparing a Factor VIII complex concentrate from blood plasma.

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1 Detailed Description

The process of this invention provides a simple and efficient purification method for high-specific-activity Factor VIII complex from an impure protein fraction. The phrase "impure protein fraction" as used herein means a solution which contains one or more protein(s) in addition to Factor VIII complex, where removal of these additional proteins is desired. The impure protein fraction used as the starting material for the purification of Factor VIII complex may be derived from a variety of sources, such as cryoprecipitate or other blood plasma-derived fractions, or it may be derived by recombinant-DNA or transgenic techniques. Briefly, the Factor VIII complex is purified by PEG precipitation, in the presence of heparin, then by chromatography on a heparin chromatography medium. The final step in the purification is to precipitate the Factor VIII complex in the presence of glycine and sodium chloride. The addition of the glycine/NaCl precipitation 1.5 to 2 fold increase in the specific activity of the Factor VIII:C complex over prior art purification methods. A detailed description of the purification procedure is set out below.

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Preparation of An Impure Protein Fraction

In one exemplary embodiment of the practice of this invention, the starting material for providing the impure protein fraction comprising Factor VIII complex is cryoprecipitate. The cryoprecipitate is recovered from human blood plasma that has been collected and tested according to procedures approved by the U.S. Food and Drug Administration. The plasma is frozen at a temperature of about -20°C, and is subsequently thawed at 0°C to 5°C. During the thawing process, a precipitate forms (the "cryoprecipitate") which is removed by centrifugation and recovered for further

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1 purification and concentration.

The cryoprecipitate is dissolved in a "heparin solution" which comprises distilled water comprising from about 30 to 150 units of heparin per ml of water at a pH of about 6.0 to about 8.5. In an exemplary embodiment, 80 units of heparin per ml of water, at pH 7.5, is used. The solution is then mixed at a temperature of from about 15°C to about 35°C until the cryoprecipitate is completely dissolved (approximately 10 minutes), to provide a cryoprecipitate/heparin solution. Preferably, the temperature during mixing is maintained at about 30°C, and the volume of heparin solution used is from about 2 to about 10 liters per kilogram of cryoprecipitate. After the cryoprecipitate is dissolved, the pH of the cryoprecipitate/heparin solution is adjusted to about 6.9 to 7.1 using, for example, 0.1M HCl, and the solution is stirred for an additional 20 to 30 minutes.

One unit of heparin is defined to mean one U.S.P. (United States Pharmacopoeia) unit. The U.S.P. unit of heparin is that quantity required to prevent 1.0 ml of citrated sheep plasma from clotting for one hour after the addition of 0.2 ml of a 1:100 calcium chloride (CaCl_2) solution. The term "heparin" as used herein is meant to include heparin itself and the pharmaceutically-acceptable, water-soluble salts of heparin, e.g., the sodium salts. A suitable example of a commercially-available heparin sodium product is U.S.P. heparin from Lyphomed Company, of Melrose Park, IL, or from Sigma Chemical Company (Sigma No. H7005), of St. Louis, MO.

Polyethylene glycol (PEG), preferably having a molecular weight in the range of from about 2000 to about 6000 (more preferably, from about 3000 to about 4000), is then added to the cryoprecipitate/heparin solution to provide a PEG solution having a final PEG concentration of from about 1% to about 5% (wt/vol).

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1 The term "% (wt/vol)" as used herein means the weight
of material added per 100 ml of starting volume of
solution. The percentages referred to herein are all
weight-per-volume, unless otherwise indicated.
5 Preferably, the PEG is added in the form of a solution
prepared by dissolving the PEG in distilled water which
comprises a citrate salt (such as sodium citrate). In
one exemplary embodiment, the aqueous PEG solution,
10 added to the cryoprecipitate/heparin solution,
comprises about 31.5% (wt/vol) PEG, 0.22% (wt/vol)
sodium citrate dihydrate, and 0.08% (wt/vol) citric
acid monohydrate at a pH of 6.2. The pH of the PEG
solution is adjusted to between 5.5 to 7.1 with an acid
such as dilute acetic acid. In one exemplary
15 embodiment, the pH is about 6.3. The pH-adjusted PEG
solution is mixed for at least about 15 minutes, at a
temperature of from 15°C to 35°C. In one embodiment,
the temperature is about 27°C.

20 The addition of 1% (wt/vol) to 5% (wt/vol) PEG
(preferably, 3% (wt/vol) to 5% (wt/vol)) to form the
PEG solution results in precipitation of various
proteins such as fibronectin and fibrinogen, leaving
Factor VIII complex in solution. The fibronectin and
other precipitated proteins, i.e., the PEG precipitate,
25 are separated from the Factor VIII complex-comprising
solution (the PEG supernatant) by centrifugation. The
PEG supernatant, i.e., the Factor VIII complex
comprising impure protein fraction, is recovered and
processed further, in accordance with the process of
30 this invention, to purify Factor VIII complex.

35 The PEG supernatant solution, i.e., the Factor
VIII complex comprising impure protein fraction, is
clarified by filtration and then further processed, for
purification of Factor VIII complex, by affinity
chromatography.

In an exemplary embodiment of practice of this
invention, the Factor VIII complex production process

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1 includes steps for inactivating viruses that may be
present in such blood products, e.g., hepatitis B
virus, hepatitis non-A/non-B virus, HIV (AIDS virus),
Cytomegalovirus, Epstein-Barr virus, and the like,
5 prior to the affinity chromatography step. In one
embodiment, a solution comprising both an organic
solvent and a detergent, is added to the PEG
supernatant to inactivate virus that may be present.
The amount of organic solvent and detergent added
10 preferably results in a solution comprising about 0.3%
(wt/vol) organic solvent and about 1% (wt/vol)
detergent. A detergent useful in practice of
principles of the invention is one sold by under the
trademark "TWEEN-80" by Fisher Scientific, of
15 Springfield, NJ; another is a detergent sold under the
trademark "TRITON X-100," by Aldrich Company, of
Milwaukee, WI. Useful organic solvents are
tri-n-butyl-phosphate (TNBP), ethyl ether, and the
like. The solution is incubated for about 6 hours to
20 about 7 hours, at a temperature of from about 24°C to
about 30°C. Inactivation of virus using organic
solvent/detergent mixture is described in U.S. Patent
No. 4,540,573, which issued on September 10, 1985 to
Neurath et al., and which is incorporated herein by
25 this reference.

Preparation of Heparin-Coupled
Chromatographic Medium

30 Preparation of the heparin-coupled chromatographic
medium is achieved in accordance with this invention by
coupling heparin or heparin sulfate to an activated
resin. Activated resins useful in the practice of this
35 invention include, but are not limited to, cyanogen
bromide-activated agarose, N-hydroxy succinimide-
activated agarose, aldehyde-activated agarose, cyanogen
bromide-activated sepharose, cyanogen bromide-activated

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1 silica, and the like.

In one exemplary embodiment of preparing a heparin-coupled chromatographic medium, heparin is bound to an activated aldehydeagarose resin supplied by Sterogene Biochemicals, of Arcadia, CA, under the trademark "ACTIGEL-A." In this embodiment, the ACTIGEL-A is washed and equilibrated in 3 volumes of a buffer, such as phosphate, acetate, or borate buffers, at concentrations of about 0.1 molar (M), and at a pH of from about 6.5 to about 7.5. A coupling mixture of heparin in a buffer comprising about 0.1M phosphate, acetate, or borate with about 0.1M sodium cyanoborohydride (NaCNBH_3), at a pH of from about 6.5 to about 7.5, is added to an equal volume of washed ACTIGEL-A resin and incubated for 12 to 20 hours at about 4°C to about 30°C with constant agitation on a mechanical mixer, such as a Labquake rotary tumbler supplied by Scientific Products, of Irvine, CA. After coupling, the mixture is filtered in a Buckner funnel, using a medium-gauge, scintered-glass filter, and the retentate, i.e., the heparin-coupled/ACTIGEL-A chromatographic medium, is washed by pouring several volumes of a solution comprising about 0.1M phosphate, acetate, or borate buffer, at about pH 6.5 to 7.5, comprising 0.5M to 1M NaCl, through the retentate while in place on the Buckner funnel. The washed heparin-coupled chromatographic medium is then incubated at 4°C to 30°C in about 0.1M ethanolamine, at about pH 6.5 to 7.5, for about two hours, to deactivate any unreacted aldehyde groups. The heparin-coupled chromatographic medium is filtered in a Buckner funnel, using a medium-gauge, scintered-glass filter, and then washed by pouring several volumes of a solution containing about 1M NaCl through the heparin-coupled chromatographic medium while in place on the Buckner funnel. Finally, the medium is washed with a buffer, such as a phosphate buffer, at a concentration of about 0.1M, at about pH

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1 6.5 to 7.5. The heparin-coupled chromatographic medium
is then stored, refrigerated at about 4°C to 10°C, in
about 0.1M phosphate buffer, pH 6.5 to 7.5, with about
0.01M sodium azide or other bactericide added as a
5 preservative.

The amount of heparin used in the coupling
reactions is preferably from about 250 to about 2000
units of heparin per ml of ACTIGEL-A, and is more
preferably about 1000 units of heparin per ml of
10 ACTIGEL-A, since this concentration gives optimal
binding of Factor VIII complex. At concentrations
below about 1000 units per ml, there are undesirably
high concentrations of Factor VIII complex found in the
chromatography effluent. At concentrations greater
15 than 1000 units of heparin per ml, there is no increase
in the amount of Factor VIII complex bound, and,
therefore, the additional heparin would add
unnecessarily to the cost of the process.

20 Preparation of The Chromatography Column

In one embodiment of practice of the process of
the invention, chromatographic columns such as those
supplied by Amicon Corporation, of Danvers, MA, are
used. The column includes an elongated, hollow
25 container having an outlet at its bottom. The heparin-
coupled chromatographic medium prepared as described
above is decanted from the sodium azide preservative
solution, in which it is stored, and washed with a
buffer, such as histidine, comprising from about 0.015M
30 to about 0.035M histidine, at a pH of about 6.5 to 7.5.
The heparin-coupled chromatographic medium is slurried
with a sufficient volume of buffer, such as 0.015M to
0.035M histidine, pH 6.5 to 7.5, so that the slurry
volume does not exceed the total column volume, and the
35 slurry is not so thick as to retain air bubbles. The
bottom of the column is filled with from about 1 to
about 3 centimeters of a solution comprising a buffer,

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1 such as 0.015M to 0.035M histidine, at about pH 6.5 to
7.5, comprising about 0.1M to 0.15M of a salt, such as
NaCl, LiCl, or KCl, at the temperature at which the
column is to be run. The slurried chromatographic
5 medium is then packed into the column by pouring it
down the side wall, to provide a heparin-coupled
chromatography column useful in the practice of this
invention to separate Factor VIII complex from the
impure protein fraction containing Factor VIII complex.

10 If desired, in accordance with the techniques of
this invention for separation of Factor VIII complex
from the impure protein fraction, the heparin-coupled
chromatographic medium can be used in a batch, rather
than a column, process. In the batch process, the
15 heparin-coupled chromatographic medium prepared as
described above is decanted from the sodium azide
solution in which it is stored and is washed with a
buffer, such as histidine, at a concentration of about
0.015M to about 0.035M, at a pH of about 6.5 to 7.5.
20 The buffer solution is decanted, and the washed
heparin-coupled chromatographic medium is added
directly to the Factor VIII complex containing impure
protein fraction.

25 Separation of Factor VIII Complex by
Affinity Column Chromatography

In an exemplary embodiment of the practice of this
invention, Factor VIII complex solution from the viral
30 inactivation step (the Factor VIII complex containing
impure protein fraction) is applied to the
chromatography column containing a heparin-coupled
chromatographic medium by pouring the solution through
the column. In a preferred embodiment of the present
35 invention, the heparin is coupled to a cross-linker
agarose resin, since the cross-linker agarose does not
compress during use to result in reduced flow rates.
While the cross-linker agarose resin is preferred,

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1 other heparin or heparin sulfate-coupled media are also
suitable for practice of this invention. Other d xtran
sulfate compounds coupled to a chromatography medium
would also be useful in the purification process. The
5 flow rate of the column is about 0.35 ml per min. for
a small (about 5 ml) column, to about 2 ml per min. for
a large (about 50 ml) column. As the impure protein
fraction flows through the column, Factor VIII complex
binds to the heparin ligand on the heparin-coupled
10 chromatographic medium, while other proteins pass
through the chromatographic medium in the column and
flow from the column as effluent. Preferably, no more
than about 20 units of Factor VIII:C activity are
applied to the column per ml of heparin-coupled
15 chromatography medium in the column when, as in one
exemplary embodiment, 1000 units of heparin are bound
per ml of activated resin. When greater than about 20
units of Factor VIII:C activity are added per ml of
heparin-coupled chromatographic medium, the excess
20 Factor VIII complex is not bound, but is instead washed
through the column into the column effluent. If less
than about 20 units of Factor VIII:C activity per ml
are added, the maximum binding capacity of the heparin-
coupled chromatographic medium (at 1000 units of
25 heparin per ml of activated resin) is not being used.

The heparin-coupled chromatographic medium with
Factor VIII complex bound to it is washed to remove all
unbound proteins. In one exemplary embodiment, the
30 washing is effected by applying about 5 to 10 volumes
of a solution comprising about 0.015M to 0.035M buffer,
such as histidine, pH 6.5 to 7.5, comprising about 0.1M
to 0.15M of a salt solution, such as LiCl, NaCl, or
KCl, and the effluent from the column is discarded.
35 Preferably, the solution comprises 0.025M histidine at
a pH of 6.8 with 0.15M NaCl. Histidine is preferred as
a buffer in the purification, since the final

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1 lyophilized Factor VIII is more easily resolubilized
when it is in a buffer comprising histidine. However,
while histidine is preferred, other buffers known in
the art could be used. Where buffers other than
5 histidine are used during the purification, it is
preferable that the final purified Factor VIII is
transferred into a buffer comprising histidine prior to
lyophilization, by dialysis or other suitable method.
The Factor VIII complex remains bound to the
10 chromatographic medium throughout the wash procedure.

Factor VIII complex is eluted from the column,
i.e., from the heparin-coupled chromatographic medium,
by applying to the column a buffered aqueous solution
incorporating calcium, magnesium, strontium, or other
15 divalent metal-ion salt, such as CaCl_2 , MgCl_2 , SrCl_2 , or
the like, and histidine. Preferably, the eluting agent
is CaCl_2 , at a concentration of from about 0.01M to about
0.3M, and about 0.015M to about 0.035M histidine. More
preferably, the CaCl_2 is at a concentration of from
20 about 0.05M to about 0.2M, and the histidine
concentration is from about 0.02M to about 0.03M, and
most preferably, the CaCl_2 is at a concentration of
about 0.1M, and the histidine concentration is about
0.025M. The pH of the solution is at a pH of from
25 about 6.0 to about 8.0. The column is washed with the
buffered CaCl_2 solution until all the Factor VIII
complex is washed from the column. Typically, from
about 2 to about 4 column-volumes of the buffered CaCl_2
solution are applied to the column to elute Factor VIII
30 complex. When the concentration of CaCl_2 is less than
about 0.05M, less than a desirable amount of Factor
VIII complex is eluted from the heparin-coupled
chromatographic medium. When the concentration of CaCl_2
is greater than about 0.2M, unwanted proteins are
35 eluted along with the Factor VIII complex, thereby
reducing the specific activity of Factor VIII complex

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1 in the final product. Also, salt concentrations
greater than 0.2M can lead to dissociation of the
Factor VIII complex, which results in the Factor VIII:C
being less stable. Preferably, the concentration of
5 CaCl₂ is about 0.1M, to maximize the amount of Factor
VIII complex eluted but to minimize elution of unwanted
proteins and dissociation of the Factor VIII complex.

10 When the concentration of histidine is greater
than about 0.035M, the high concentrations are wasteful
of the histidine. Histidine concentrations less than
about 0.015M have insufficient buffering capacity to
ensure that the pH remains at the desired level.

15 The Factor VIII complex eluted from the heparin-
coupled chromatographic medium is concentrated 10 to 15
fold by ultra-filtration, using an ultrafilter (or its
equivalent) such as that supplied under the trade name
of "CENTRASSETTE" Omega 100K cassette, by Filtron.

20 Separation of Factor VIII Complex by
Affinity Chromatography in a Batch Process

25 In an exemplary embodiment of the practice of this
invention, Factor VIII complex solution from the viral
inactivation step (the Factor VIII complex containing
impure protein fraction) is applied directly to the
washed heparin-coupled chromatographic medium and mixed
for about 30 min. to about 45 min. for batch
processing. During this time, the Factor VIII complex
binds to the heparin ligand on the chromatographic
30 medium, leaving a supernatant containing proteins other
than Factor VIII complex in solution. The
chromatographic medium is removed by decanting the
supernatant, and the medium is then washed to remove
unbound proteins. In one exemplary embodiment, the
35 washing is effected by resuspending the Factor VIII
complex-bound, heparin-coupled chromatographic medium
in about 5 to 10 volumes of a solution comprising about

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1 0.015M to 0.035M buffer, such as histidine, pH 6.5 to
7.5, comprising a salt solution, such as LiCl, NaCl, or
KCl, at a concentration of about 0.1M to about 0.15M.
Preferably, the solution comprises 0.025M histidine at
5 a pH of 6.8 comprising 0.15M NaCl. The Factor VIII
complex-bound, heparin-coupled chromatographic medium
is removed from the wash solution by decanting the
supernatant, i.e., the wash solution. The Factor VIII
complex remains bound to the heparin-coupled
10 chromatographic medium throughout the wash procedure.

Factor VIII complex is eluted from the heparin-
coupled chromatographic medium by applying to the
medium a buffered aqueous solution incorporating
calcium, magnesium, strontium, or other divalent metal-
ion salt, such as CaCl₂, MgCl₂, SrCl₂, or the like, and
histidine. Preferably, the eluting agent is CaCl₂ at a
concentration of from about 0.01M to about 0.3M, and
about 0.015M to about 0.035M histidine. More
preferably, the CaCl₂ is at a concentration of from
15 about 0.05M to about 0.2M, and the histidine
concentration is from about 0.02M to about 0.03M, and
most preferably, the CaCl₂ is at a concentration of
about 0.1M, and the histidine concentration is about
0.025M. The solution is at a pH of from about 6.0 to
20 about 8.0. The medium is washed with the buffered CaCl₂
solution by resuspending the medium in the buffer and
separating the medium from the buffer by
centrifugation. The wash is repeated until
25 substantially all of Factor VIII:C is recovered from
the heparin chromatography medium. When the
concentration of CaCl₂ is less than about 0.05M, less
than a desirable amount of Factor VIII complex is
eluted from the heparin-coupled chromatographic medium.
When the concentration of CaCl₂ is greater than about
30 0.2M, unwanted proteins are eluted along with the
Factor VIII complex, thereby reducing the specific
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1 activity of Factor VIII complex in the final product.
Also, salt concentrations greater than 0.2M can lead to
dissociation of the Factor VIII complex, which results
in the Factor VIII:C being less stable. Preferably,
5 the concentration of CaCl₂ is about 0.1M, to maximize
the amount of Factor VIII complex eluted but to
minimize elution of unwanted proteins and dissociation
of the Factor VIII complex.

10 The Factor VIII complex eluted from the heparin-
coupled chromatographic medium is washed by ultra-
filtration, using an ultrafilter (or its equivalent)
such as that supplied under the trade name of
"CENTRASSETTE" Omega 100K cassette.

15 Purification of Factor VIII complex
by Glycine/NaCl Precipitation

20 The Factor VIII complex in the ultrafiltered
material is precipitated by the addition of glycine and
NaCl. The glycine/NaCl is an effective means of
increasing the specific activity of the Factor VIII
complex. However, it is important that this step be
included after the heparin chromatography step, or
other suitable partial purification step, since its
inclusion at an earlier step results in a "milky"
25 solution, thereby causing the precipitation of
undesirable components from the solution, in addition
to Factor VIII. The fractions recovered from the
heparin chromatography medium are brought to about 1.5M
to about 2.5M glycine and about 1M to about 2M NaCl.
30 The solution is mixed at about 15° to about 25°C for
about two hours, and the precipitate which forms is
collected by centrifugation. Preferably, 2M glycine
and 1.3M NaCl are added to the fractions recovered from
the heparin chromatography medium.

35 The precipitate, which contains Factor VIII
complex, is washed with a wash solution comprising

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1 about 0.025M histidine, pH 6.8, 2M glycine, and 1.3M
NaCl, and is collected by centrifugation. The wash
procedure results in the resolubilization of
contaminating proteins caught in the glycine/NaCl
5 precipitate but not the Factor VIII complex. The wash
procedure is then repeated, if desired, and the final
precipitate is collected by centrifugation or
filtration and dissolved in a buffer comprising about
0.025M histidine, 0.1M arginine, pH 6.0 to 8.0. After
10 the precipitate is dissolved, about 0.02 to about 1%
(wt/vol) albumin is added as a bulking agent if
desired, and the solution is filtered.

The solution is then divided among separate vials,
with each vial containing a desired number of units of
15 Factor VIII:C activity. The solutions are then
lyophilized to provide separate vials of purified
Factor VIII complex concentrate.

Example 1

20 Preparation of an Impure Protein
Fraction Containing Factor VIII

Forty grams of cryoprecipitate was dissolved in
120 ml of distilled water containing about 80 units of
heparin per ml of water. The heparin solution was
25 mixed at a temperature of about 30°C until the
cryoprecipitate was completely dissolved (approximately
10 minutes), to provide a cryoprecipitate/heparin
solution. After the cryoprecipitate was dissolved, the
pH of the cryoprecipitate/heparin solution was
30 adjusted to about pH 7.0 using 0.1M HCl, and the
solution was stirred for an additional 20 to 30
minutes.

An aqueous PEG solution comprising about 31.5%
(wt/vol) PEG, 0.22% (wt/vol) sodium citrate dihydrate,
35 and 0.08% (wt/vol) citric acid monohydrate, at a pH of
6.2, was then added to the cryoprecipitate/heparin
solution to give a final concentration of 3.5% (wt/vol)

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1 PEG. The pH of the PEG/cryoprecipitate/heparin
solution was adjusted to about 6.3 with dilute acetic
acid. The pH-adjusted solution was mixed for
approximately 15 minutes, at a temperature of about
5 27°C. The addition of PEG resulted in precipitation of
various contaminating proteins from the Factor VIII
complex which remained in solution.

10 The PEG precipitate was separated from the Factor
VIII complex-containing supernatant solution by centri-
fugation. The supernatant, i.e., the Factor VIII
complex containing impure protein fraction, was
recovered. The supernatant was then treated to
inactivate viruses which may be present in the blood
products, by adding a solution containing about 0.3%
15 (wt/vol) tri-n-butylphosphate and about 1% (wt/vol)
TWEEN-80, and incubating at 25°C for about 6 hrs.

20 The viral-inactivated supernatant solution, i.e.,
the viral-inactivated Factor VIII complex containing
impure protein fraction, was clarified by filtration,
and was then recovered for further purification of
Factor VIII complex by affinity chromatography on a
heparin-coupled chromatographic medium.

Example 2

25 Purification of Factor VIII Complex
by Heparin Affinity Chromatography

An impure protein fraction prepared by a process
such as the process described in Example 1, containing
a total of 1,000 units of Factor VIII:C activity, was
30 applied to heparin-coupled chromatography medium packed
into a column, and the flow rate of the column was
maintained at 2 ml per min. The column effluent was
collected, and the column was washed with 600 ml of
0.025M histidine, pH 6.8, containing 0.15M NaCl.
35 Elution of Factor VIII complex was achieved with 200 ml
of 0.1M CaCl₂ and 0.025M histidine, pH 6.8. All
effluent and eluate samples are assayed for Factor

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- 1 VIII:C blood-clotting activity using a COAG-A-MATE XC
clotting machine.

Example 3

5 Glycine/NaCl Precipitation of Factor VIII

Factor VIII complex prepared in accordance with a process such as that shown in Example 2 was concentrated using a "CENTRASSETTE," Omega 100K cassette. The concentrated solution was then brought 10 to 2M glycine and 0.83M NaCl and mixed at 25°C for 2 hours. The glycine/NaCl precipitate which formed was collected by centrifugation and washed with 100 ml of a wash solution comprising 0.025M histidine, pH 6.8, 2M glycine, and 1.3M NaCl. The washed precipitate was 15 again collected by centrifugation, and the wash procedure was repeated. The final washed precipitate was dissolved in 10 ml buffer comprising about 0.025M histidine, 0.1M arginine, pH 7.0 to 7.6. After the precipitate was dissolved, 0.2% (wt/vol) albumin was 20 added, and the solution was filtered. The resultant solution was then assayed for Factor VIII:C blood clotting activity using a COAG-A-MATE XC clotting machine. The concentration of fibronectin in the final precipitate was also analyzed. The results are 25 summarized in Table I.

Example 4

The procedure of Example 3 was repeated, except that 2M glycine and 1.3M NaCl were used to form the 30 glycine/NaCl precipitate. The resultant solution was then assayed for Factor VIII:C blood clotting activity using a COAG-A-MATE XC clotting machine. The concentration of fibronectin in the final precipitate was also analyzed. The results are summarized in Table 35 I.

Example 5

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1 The procedure of Example 3 was repeated, except
 that 2M glycine and 1.38M NaCl were used to form the
 glycine/NaCl precipitate. The resultant solution was
 then assayed for Factor VIII:C blood clotting activity
 5 using a COAG-A-MATE XC clotting machine. The
 concentration of fibronectin in the final precipitate
 was also analyzed. The results are summarized in Table
 I.

10

Example 6

15 The procedure of Example 3 was repeated, except
 that 2M glycine and 1.56M NaCl were used to form the
 glycine/NaCl precipitate. The resultant solution was
 then assayed for Factor VIII:C blood clotting activity
 15 using a COAG-A-MATE XC clotting machine. The
 concentration of fibronectin in the final precipitate
 was also analyzed. The results are summarized in Table
 I.

20

Example 7

25 The procedure of Example 3 was repeated, except
 that 2M glycine and 1.93M NaCl were used to form the
 glycine/NaCl precipitate. The resultant solution was
 then assayed for Factor VIII:C blood clotting using a
 COAG-A-MATE XC clotting machine. The concentration of
 fibronectin in the final precipitate was also analyzed.
 The results are summarized in Table I.

Table I

	% Yield in Precipitate ¹ Factor VIII	% Yield in Precipitate Fibronectin	Specific Activity Factor VIII ²
30	0.83M NaCl*	2	<1
	1.30M NaCl	62	2
	1.38M NaCl	103	4
35	1.56M NaCl	91	14
	1.93M NaCl	95	28

* The experiments were performed in the presence of

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- 1 the NaCl concentration indicated and 2M glycine.
- 1 ¹ % yield in the precipitate is the activity of Factor VIII:C in the precipitate divided by the activity of Factor VIII:C contained in the cryoprecipitate multiplied by 100.
- 5 ² Factor VIII:C units/mg protein.

Example 8

10 After 9,030 kg of plasma were cryoprecipitated, the resultant 107 kg of cryoprecipitate was dissolved in 320 l of distilled water containing about 120 units of heparin per ml of water. The heparin solution was mixed at a temperature of about 30°C until the cryoprecipitate was completely dissolved (approximately 15 min.), to provide a cryoprecipitate/heparin solution. After the cryoprecipitate was dissolved, the pH of the cryoprecipitate/heparin solution was adjusted to about 7 using 0.1M HCl, and the solution was stirred for an additional 20 to 30 min.

20 An aqueous PEG solution comprising about 31.5% (wt/vol) PEG, 0.22% (wt/vol) sodium citrate dihydrate, and 0.08% (wt/vol) citric acid monohydrate, at a pH of 6.2, was then added to the cryoprecipitate/heparin solution to give a final concentration of 3.5% (wt/vol) PEG. The pH of the PEG/cryoprecipitate/heparin solution was adjusted to about 6.3 with dilute acetic acid. The pH-adjusted solution was mixed for approximately 15 minutes, at a temperature of about 27°C. The addition of PEG resulted in precipitation of various contaminating proteins from the Factor VIII complex, which remained in solution.

35 The PEG precipitate was separated from the Factor VIII complex-containing supernatant solution by centrifugation. The PEG supernatant, i.e., the Factor VIII complex containing impure protein fraction, was recovered. The supernatant was then treated to inactivate viruses which may be present in the blood

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1 products, by the addition of a solution containing about 0.3% (wt/vol) tri-n-butylphosphate and about 1% (wt/vol) TWEEN-80 and incubating at 25°C for about 6 hrs.

5 The viral-inactivated supernatant solution, i.e., the viral-inactivated Factor VIII complex containing impure protein fraction, was clarified by filtration and then recovered for further purification of Factor VIII complex by affinity chromatography on a heparin-coupled chromatographic medium.

10 The Factor VIII complex-containing solution was applied to a 200 liter (1) heparin-coupled chromatographic medium packed into the column. The column effluent was collected, and the column was washed with 1700 l of 0.025M histidine, pH 6.8, containing 0.10M NaCl. Elution of Factor VIII complex was achieved with 600 l of 0.1M CaCl₂ and 0.025M histidine, pH 6.8.

15 The eluate from a heparin column (the column eluate) was concentrated 15 fold using a CENTRASSETTE, Omega 100K cassette. The concentrated solution, i.e. the eluate concentrate, was then brought to 2M glycine and 1.2M NaCl and mixed at 25°C for 2 hours. The precipitate which formed was collected by centrifugation and washed with a wash solution comprising 0.025M histidine, pH 6.8, 2M glycine, and 1.3M NaCl. The washed precipitate was collected by filtration. The washed precipitate was dissolved in 2 l buffer comprising about 0.025M histidine, 0.1M arginine, pH 7.0 to 7.6. After the precipitate was dissolved, 0.5% (wt/vol) albumin was added, and the solution was filtered.

20 The resultant solution was then assayed for Factor VIII:C blood clotting activity using a COAG-A-MATE XC clotting machine. The results are summarized in Table III.

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Table II

	<u>Sample</u>	<u>Units¹ x 10⁻³</u>	<u>Units/kg Plasma</u>	<u>Specific Activity units/mg</u>
5	Plasma	9,030	1,000	0.01
	Cryoprecipitate	3,540	392	0.7
	PEG-supernatant	3,645	404	1.5
	Column eluate	2,640	292	-
	Eluate Conc.	1,540	171	14.5
	Glycine/NaCl	782	87	99.1
				(16.5)*

10 * Specific activity after addition of HSA.

1 Units of Factor VIII:C activity.

15 The resultant purified Factor VIII complex solution was further analyzed to evaluate the contaminating proteins present. The results are summarized in Table III.

Table III

20	Specific Activity (Factor VIII:C units/mg)	99.1
	Fibronectin (μ g/unit*)	1.5
	Fibrinogen (μ g/unit)	<0.8
	IgG (μ g/unit)	<0.1
	IgM (μ g/unit)	\leq 0.1
	HSA (μ g/unit)	<0.1

25 * per unit of Factor VIII:C.

Example 9

Comparison of Various Preparations of Factor VIII

30 A number of Factor VIII complex preparations were made in accordance with the procedure of Example 8. The purified samples were analyzed for Factor VIII:C specific activity and also for contamination with fibronectin, fibrinogen and IgM. The results obtained from these purifications are summarized in Table IV.

35

Table IV

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1 Lot No.	<u>Factor VIII:C</u>		Fibro- nectin μg/unit*	Fibrin- ogen μg/unit*	IgM μg/unit*
	Yield units/kg	Specific Activity ¹ units/mg ²			
5	1	194	68.8	0.5	3.8 0.13
	2	164	77.3	0.53	<0.5 <0.02
	3	174	91.9	0.48	0.44 <0.02
	4	178	110.2	0.43	<0.66 <0.03
	5	140	108.0	n.m.	n.m. n.m.

* per unit of Factor VIII

10 n.m. not measured

¹ Factor VIII:C/mg protein.² The variation in the specific activity level is due to inexperience with and the development of the new purification for Factor VIII. Specific activities of at least 100 are now routinely obtained.

The above descriptions of exemplary embodiments of processes for producing Factor VIII complex concentrates are for illustrative purposes. Because of variations which will be apparent to those skilled in the art, the present invention is not intended to be limited to the particular embodiments described above. This invention can also be practiced in the absence of any element not specifically disclosed. The scope of the invention is defined by the following claims.

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1 WHAT IS CLAIMED IS:

5 1. A process for separating Factor VIII complex from an impure protein fraction containing Factor VIII complex, the process comprising the steps of:

providing an aqueous solution of an impure protein fraction comprising Factor VIII complex;

10 applying the impure protein fraction solution to a heparin-coupled chromatographic medium to thereby bind Factor VIII complex to the heparin;

eluting the Factor VIII complex from the chromatographic medium using an aqueous solution comprising CaCl_2 ;

15 adding a sufficient amount of glycine and sodium chloride to the eluate to thereby precipitate Factor VIII complex;

washing the Factor VIII complex precipitate with a wash solution; and

recovering the washed Factor VIII complex.

20

2. The process of claim 1 wherein the CaCl_2 is present in the solution at a concentration of from about 0.01M to about 0.3M.

25

3. The process of claim 1 wherein the aqueous CaCl_2 solution further comprises histidine at a concentration of from about 0.015M to about 0.035M.

30

4. The process of claim 3 wherein the concentration of histidine is about 0.025M.

5. The process of claim 3 wherein the aqueous CaCl_2 solution has a pH of from about 6.0 to 8.0.

35

6. The process of claim 5 wherein the pH is maintained at about 6.8.

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- 1 7. The process of claim 1 wherein the Factor
VIII complex is precipitated from the eluate with about
1.5M to about 2M glycine and about 1M to about 2M
sodium chloride.
- 5 8. The process of claim 1 wherein the Factor
VIII complex is precipitated from the eluate with about
2M glycine and about 1.3M sodium chloride.
- 10 9. The process of claim 1 wherein the wash
solution comprises about 0.025M histidine, pH 6.8, 2M
glycine, and 1.3M sodium chloride.
- 15 10. The process of claim 1 wherein the impure
protein fraction containing Factor VIII complex is
derived from cryoprecipitate.
- 20 11. The process of claim 1 wherein the impure
protein fraction is applied to the heparin-coupled
chromatographic medium in a batch process.
- 25 12. The process of claim 1 wherein the recovered
Factor VIII complex precipitate has a specific
activity, with respect to the Factor VIII:C activity,
of about 70 to 150 units/mg.

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1 13. A process for separating Factor VIII complex from an impure protein fraction containing Factor VIII complex, comprising the steps of:

5 providing an aqueous solution of an impure protein fraction containing Factor VIII complex;

 applying the impure protein fraction solution to a chromatographic medium comprising a heparin ligand coupled to a resin;

10 binding Factor VIII complex to the heparin ligand on the chromatographic medium;

 eluting the Factor VIII complex from the chromatographic medium using an aqueous solution comprising CaCl_2 as the eluting agent;

15 precipitating the Factor VIII complex from the eluate with glycine and sodium chloride;

 washing the precipitate with a wash solution; and

20 dissolving the Factor VIII complex precipitate in an aqueous solution to provide a Factor VIII complex solution with a specific activity, with respect to the Factor VIII:C activity, of about 70 to about 150 units/mg.

25 14. The process of claim 13 wherein the concentration of CaCl_2 in the aqueous solution is from about 0.05M to about 0.2M.

30 15. The process of claim 13 wherein the aqueous CaCl_2 solution has a pH of from about 6.0 to 8.0.

 16. The process of claim 15 wherein the pH is maintained at about 6.8.

35 17. The process of claim 13 wherein the aqueous CaCl_2 solution includes histidine at a concentration of from about 0.015M to about 0.035M.

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1 18. The process of claim 17 wherein the concentration of histidine is about 0.025M.

5 19. The process of claim 13 wherein the Factor VIII complex is precipitated with about 1.5M to about 2M glycine and about 1M to about 2M sodium chloride.

10 20. The process of claim 13 wherein the Factor VIII complex is precipitated with about 2M glycine and about 1.3M sodium chloride.

15 21. The process of claim 13 wherein the wash solution comprises about 0.025M histidine, pH 6.8, 2M glycine, and 1.3M sodium chloride.

22. The process of claim 13 wherein the impure protein fraction containing Factor VIII is derived from cryoprecipitate.

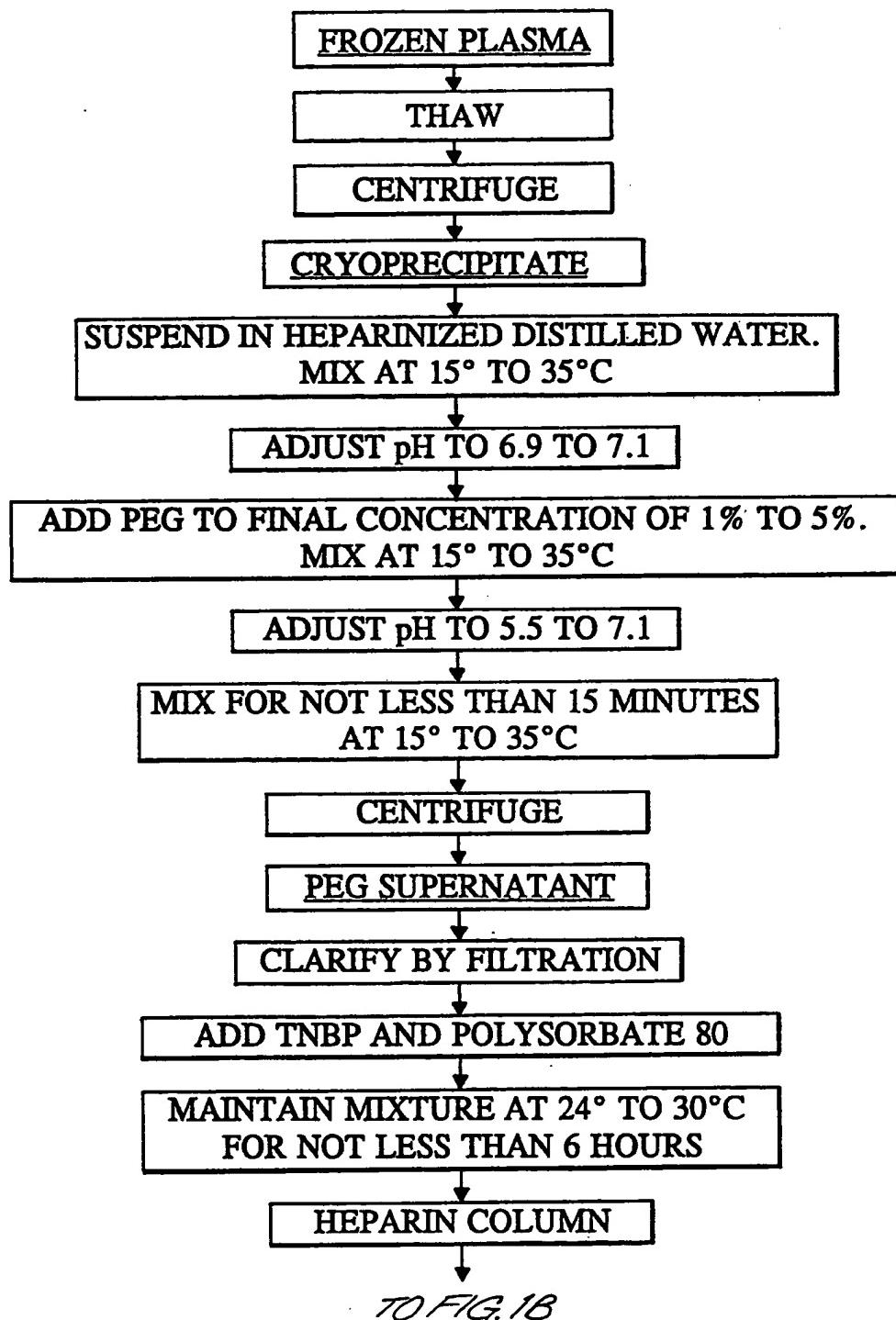
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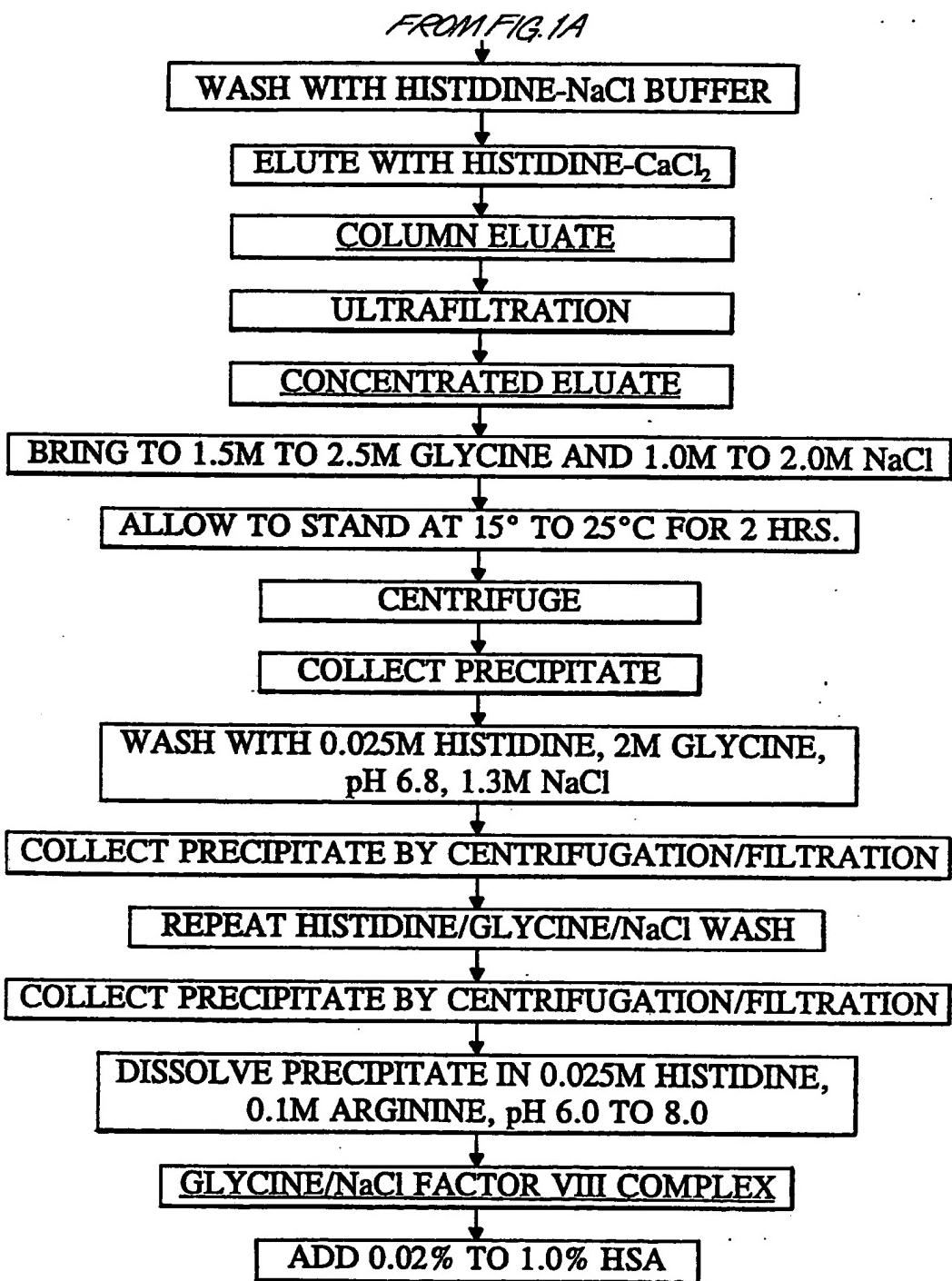
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1/2
FIG. 1A



2/2
FIG. 1B

INTERNATIONAL SEARCH REPORT

International application No. PCT/US93/04058

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) :C07K 3/20; A61K 35/14; A61K 37/02; C07K 13/00
US CL :530/383; 530/413, 415, 419, 420

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/383; 530/413, 415, 419, 420

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,758,657 (Farb et al.) 19 July 1988, column 4, lines 24-63.	1-22
X,P	US, A, 5,110,907 (Kosow et al.) 05 May 1992, column 3, line 14, claims 6 and 9.	1-22
Y	US, A, 4,543,210 (Mitra et al.) 24 September 1985, see entire document.	1-22

Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

26 JULY 1993

Date of mailing of the international search report

16 AUG 1993

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